PROLINE IN FASCIOLIASIS: II. CHARACTERISTICS OF PARTIALLY PURIFIED ORNITHINE - δ - TRANSAMINASE FROM *FASCIOLA*

by Jeanette C. Ertel and Hadar Isseroff

ABSTRACT

Ornithine-δ-transaminase from *Fasciola* was partially purified by differential centrifugation and precipitation by ammonium sulfate. The K_m's for the purified transaminase were 2.33 mM and 2.24 mM for ornithine and α-ketoglutarate, respectively. The pH optimum (8.0) and the inhibitory effect of other amino acids is similar to that of the mammalian enzyme. Pyridoxal dependence and inhibition by pyridoxal inhibitors were also demonstrated. The only observed peculiarity of the fluke enzyme that might explain its high activity appears to be a high tolerance for α-ketoglutarate.

INTRODUCTION

Recent studies have shown that free proline occurs in high levels in *Fasciola hepatica* (Kurelec and Rijavec, 1966) and in the bile of animals infected with this trematode (Isseroff et al., 1972). Ornithine-δ-transaminase [E.C. 2.6.13 L-ornithine: 2-oxoacid aminotransferase], hereafter called OTA, catalyzes the formation of the immediate precursor to proline, Δ^1-pyrroline-5-carboxylic acid, from ornithine and α-ketoglutarate. In a previous paper (Ertel and Isseroff, 1974) this enzyme was shown to occur in homogenates of *Fasciola* and to have seven and ten times the activity of the OTA in rat and rabbit liver, respectively. It was the high activity of the OTA, coupled with the lack of an enzyme capable of breaking down proline, which explained the high levels of proline in the worm. These data also suggested that the excessive proline in the biliary fluid of infected animals originated in the worm. The present study deals with some of the properties of this enzyme in partially purified preparations from *Fasciola.*

Jeanette Ertel is a Research Associate at SUNY College at Buffalo. Hadar Isseroff is Associate Professor of Biology at SUNY College at Buffalo.

METHODS AND MATERIALS

Preparation of ornithine-δ-transaminase enzyme

Infections of *Fasciola hepatica* were maintained in New Zealand rabbits and Wistar rats according to the methods of Isseroff and Read (1969). Rabbits or rats with 75- to 90-day infections were killed to obtain the worms. After removal from the bile ducts, they were rinsed in ice cold 0.05M tris-chloride buffer, pH 8.0, blotted on filter paper, and weighed. For homogenization, buffer was added in the proportion of 10 ml/g of fluke tissue, and the process was carried out at 0° C for 5 minutes in a Potter-Elvehjem homogenizer. The following procedure was carried out at 0° C. The homogenate was centrifuged at 12,000 × *g* for 20 minutes and the supernatant was removed. Sufficient ammonium sulfate (Fisher Chem. Co.) was then added to achieve 60% saturation. After standing for 30 minutes, the resulting suspension was centrifuged at 3000 × *g* for 10 additional minutes before being recentrifuged at 3000 × *g* for 10 minutes. The partially isolated enzyme was dissolved in a small amount of 0.05M tris-chloride buffer, pH 8.0. This was dialyzed in the cold against two changes of water for 2 hours and subsequently lyophilized.

Enzyme Assays

Δ^1-pyrroline-5-carboxylic acid (PCA), the product of the OTA reaction, was assayed by Strecker's method as in a previous study (Ertel and Isseroff, 1974) but with the following modification: the concentration of α-ketoglutarate was increased to 20 mM (except where indicated) because this concentration was found to be saturating and non-inhibitory to the reaction. Enzyme assays were carried out with three or more samples per experiment. We used reagents of the highest purity available, purchased from the Sigma Chemical Company unless another source is indicated. Specific activity, or V_{max}, is defined as μmoles of product/mg of protein per hour. Protein was determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard. In determining K_m's the concentration of protein was approximately 0.01 mg/ml of incubation mixture. This concentration was found to be well within the linear range when velocity was measured as a function of protein concentration using 35 mM ornithine and 20 mM α-ketoglutarate. Time-course studies showed that the reaction was linear for at least 30 minutes with these concentrations of enzyme and substrates.

Determination of α-ketoglutarate

For determination of α-ketoglutarate levels in *Fasciola*, the worms were removed from the bile ducts of infected rats killed by etherization, then were quickly blotted on filter paper and weighed. A number of flukes giving an approximate wet weight of 0.6g were immediately placed in 4.0 ml of 0.6M perchloric acid and homogenized in a Potter-Elvehjem homogenizer. One ml of acid was used to wash out the tube and the precipitated protein was

removed by centrifugation. Perchloric acid was then removed from the supernatant by adding 1.0M K_2CO_3 and the extract adjusted to pH 7.2. Liver samples were obtained from healthy rats dispatched with ether and treated similarly except that 0.5 to 1.0 g wet weight of tissue was used. The concentration of α-ketoglutarate was then tested by an enzymatic assay using glutamic dehydrogenase (Bergmeyer and Bernt, 1965).

RESULTS

The specific activity of the 60-75% fraction was four to five times that of the crude homogenate. Table 1 shows a typical purification of OTA from two grams wet weight of fluke material, with an approximate yield of 20%.

Previous studies in our laboratory have shown the pH optimum for OTA in crude homogenates of *Fasciola*, rat liver, and rabbit liver to be 8.0. A similar pH optimum was previously reported for rat liver (Katunuma et al., 1964) and pig kidney (Jenkins and Tsai, 1970). In the present study with 0.025M tris-chloride buffer, the purified enzyme also showed maximal activity at pH 8.0 (figure 1). All subsequent experiments were, therefore, carried out in 0.025M tris-chloride buffer, pH 8.0.

Figure 2 shows the effect of temperature on OTA. In the presence of ornithine, α-ketoglutarate, and pyridoxal phosphate, the optimum temperature for 30-minute incubations was found to be 45°C, with a sharp drop in activity above 50°C.

The Michaelis constants were determined for each of the substrates, ornithine and α-ketoglutarate, according to the methods of Lineweaver and Burk (1934). With preparations having specific activities of about 19-26 μmoles of PCA/mg of protein per hour, the K_m values for ornithine and α-ketoglutarate were determined to be 2.33 and 2.24 mM, respectively (table 2 and figures 3 and 4).

TABLE 1

COMPARISON OF ACTIVITIES AND YIELDS OF OTA IN THE CRUDE HOMOGENATE AND AFTER PARTIAL PURIFICATION

Fraction	Total units	Specific activity	Purification	% Yield
crude homogenate	661	5.8	1	100
ammonium sulfate fraction (60-75%)	133	25.0	4.3	20

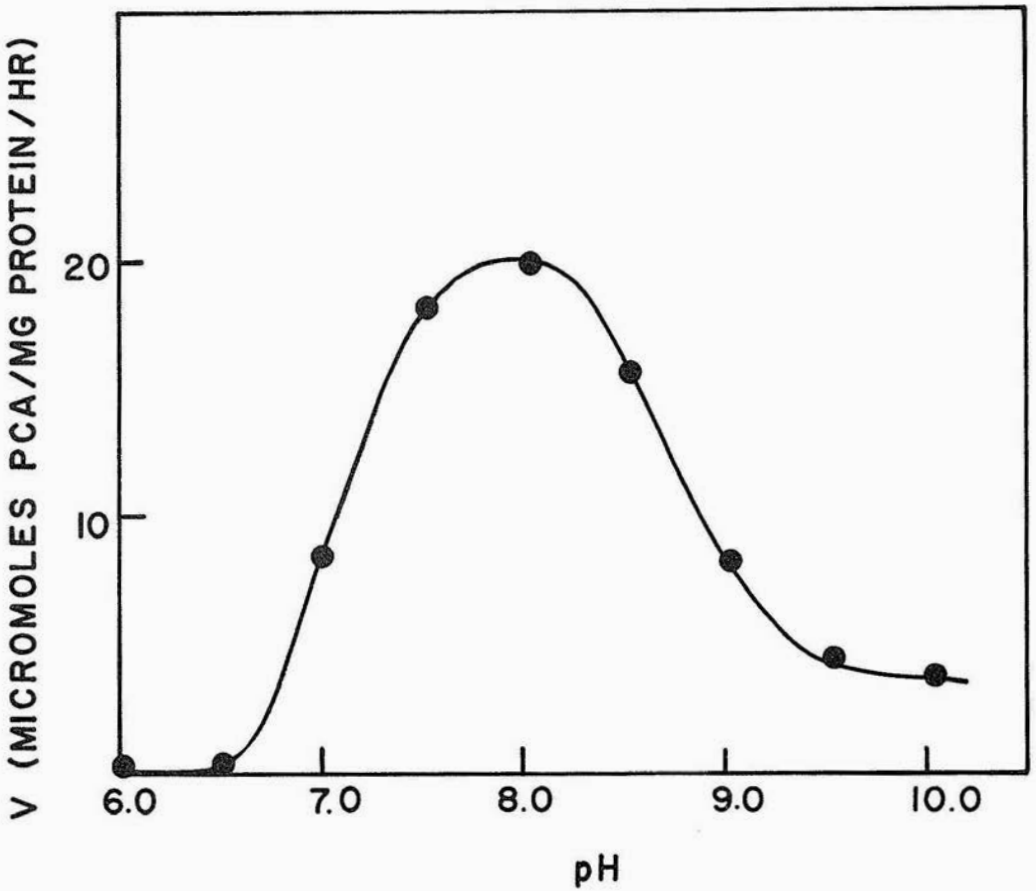

FIG. 1. The effect of pH on the activity of OTA, with 0.025M Tris Cl buffer. Each point is the mean of three samples.

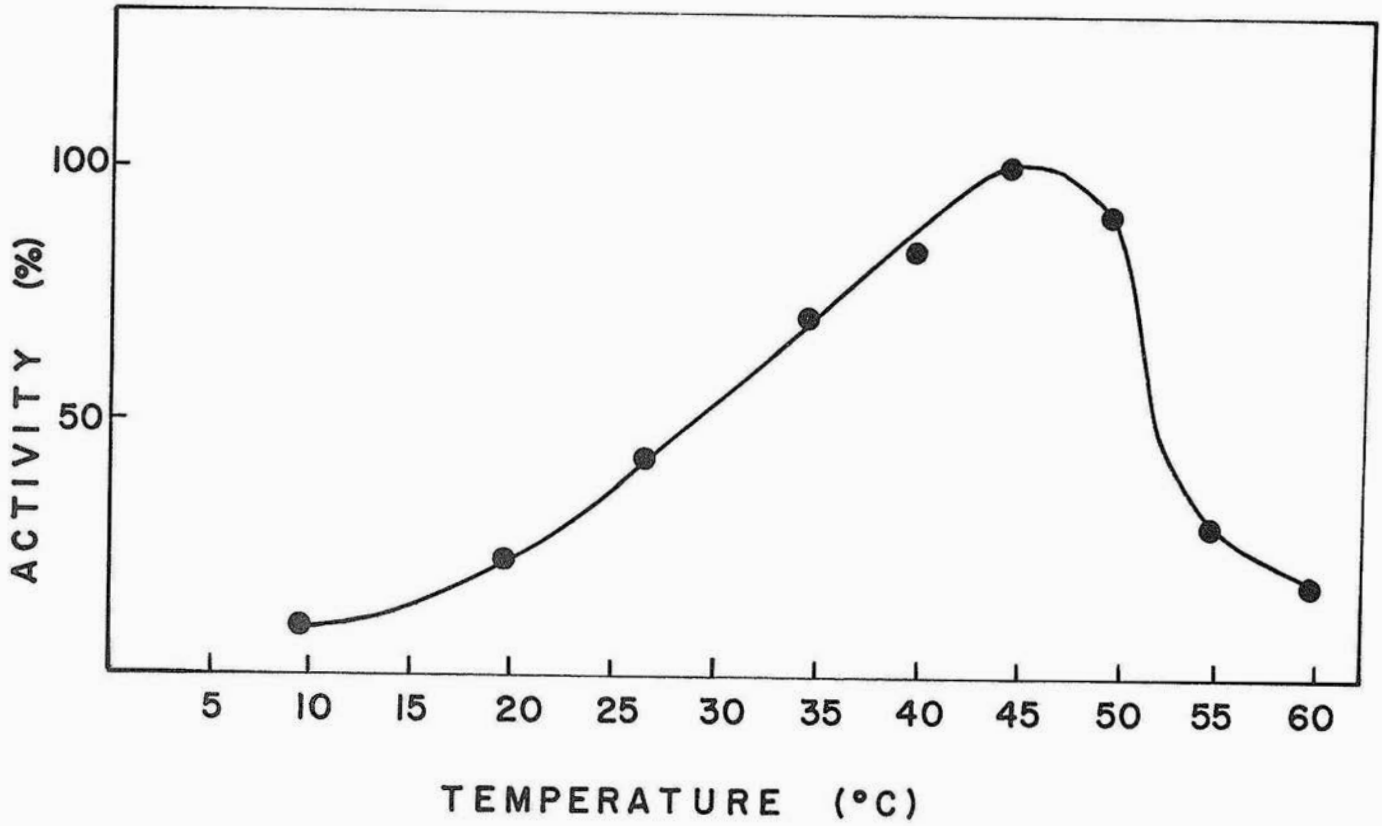

FIG. 2. The effect of temperature on OTA activity in 30 minute incubations. Each point is the mean of five samples.

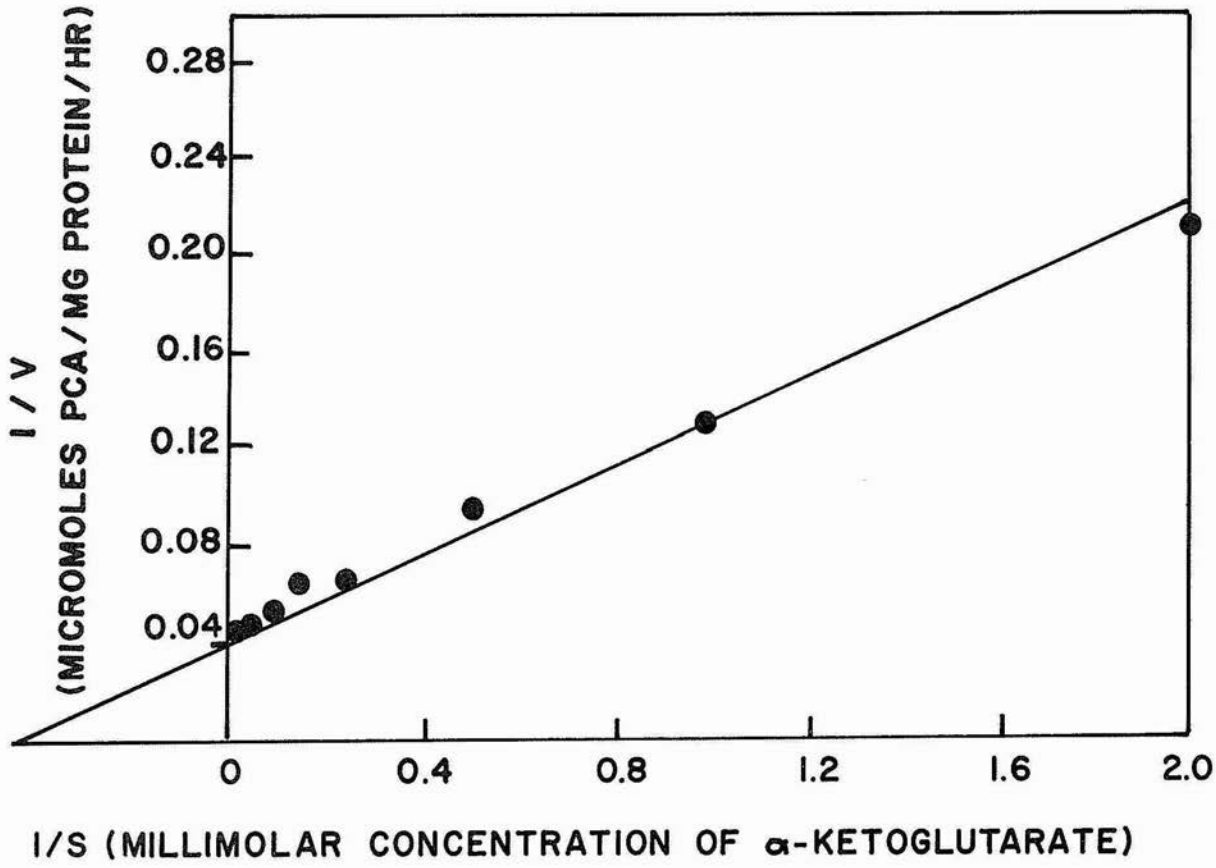

FIG. 3. Typical Lineweaver-Burk plot of ornithine concentration versus velocity of OTA reaction. Each point is the mean of three samples.

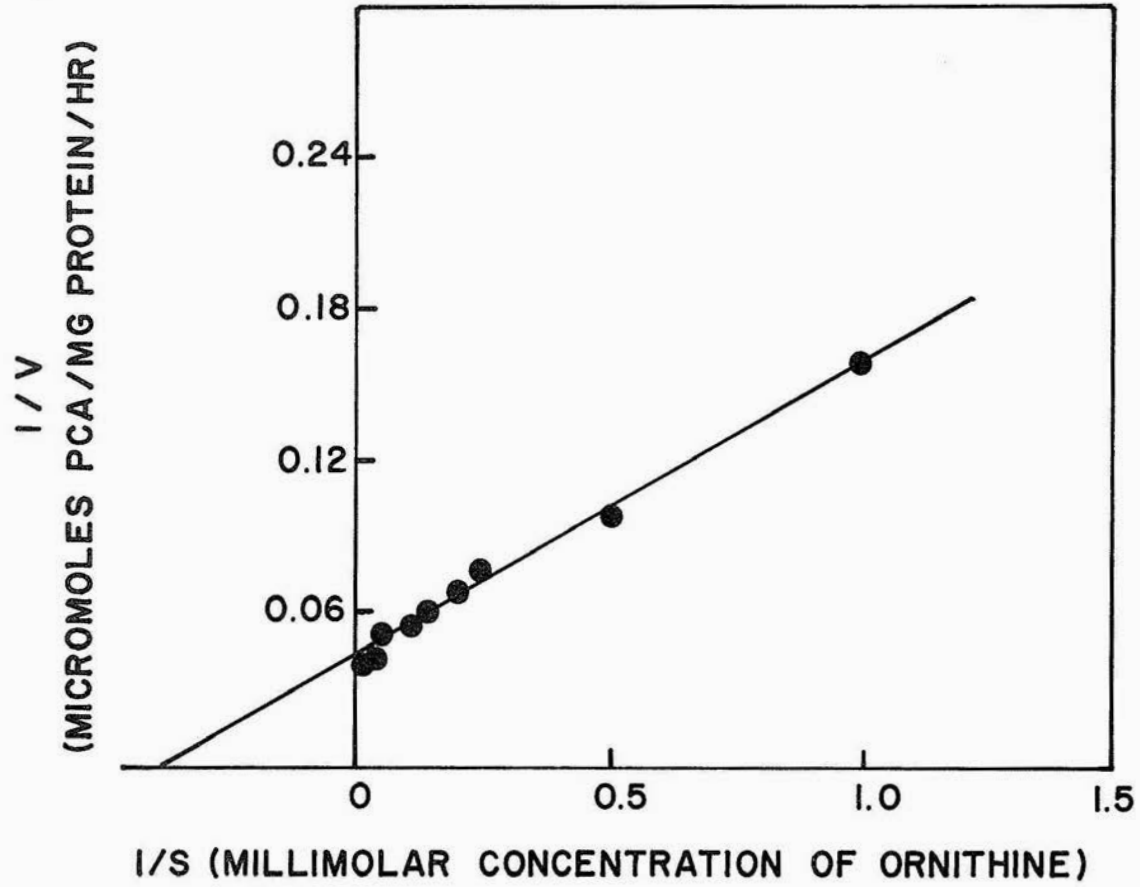

FIG. 4. Typical Lineweaver-Burk plot of α-ketoglutarate concentration versus velocity of OTA reaction. Each point is the mean of three samples.

TABLE 2

K_m AND V_{max} VALUES FOR OTA

K_m values are in μmoles of ornithine or α-ketoglutarate per ml. V_{max} values are in μmoles of PCA produced/mg of protein per hour. Ornithine was present at 35 mM for K_m determinations of α-ketoglutarate. α-ketoglutarate was present at 10 mM for K_m determinations of ornithine. Values shown are the means of three experiments.

Substrate	$K_m \pm$ S. E.	$V_{max} \pm$ S. E.
ornithine	2.33 ± 0.229	18.7 ± 4.001
α-ketoglutarate	2.24 ± 0.198	26.2 ± 1.217

TABLE 3

EFFECT OF PYRIDOXAL-5-PHOSPHATE ON THE ACTIVITY OF ORNITHINE-δ-TRANSAMINASE

Ornithine and α-ketoglutarate were present at 35.0 and 20.0 μmoles/ml, respectively. The pH was 8.0 in all vessels. Each value is the mean of three determinations. The effect given is significant at $P < 0.01$ in a two tailed Student's t test.

Addition of pyridoxal PO_4	μmoles of PCA produced/mg of protein/hour ± S.E.	Significant effect (%)
none	8.79 ± 0.123	—
2.5 μmoles/ml	17.93 ± 0.520	+104

Although the crude homogenate did not require added pyridoxal phosphate, the purification process resulted in decreased activity unless this cofactor was added (table 3). Moreover, the absolute requirement for the cofactor is indicated by the effects of pyridoxal inhibitors as shown in table 4. At 25 mM, hydroxylamine, thiosemicarbazide, INAH (isonicotinamide hydrazide), and D-cycloserine all caused significant inhibition.

The effect of compounds structurally related to ornithine on the activity of OTA was also determined. Significant inhibition was caused by norvaline, δ-aminovaleric acid, and α-ketovaleric acid. Inhibitory effects by hydroxyproline, isoleucine, leucine, proline, and valine were not detected (table 4).

Since the optimum concentration for α-ketoglutarate in the fluke OTA reaction was much higher than that of rat liver (Strecker, 1965) and rabbit liver (Ertel and Isseroff, unpublished), the α-ketoglutarate concentration in *Fasciola* was determined. In *Fasciola* the level of α-ketoglutarate was found to be about 214 nanomoles/g wet weight (table 5), or approximately 4.0 × greater than the concentration of that compound in rat liver.

TABLE 4

EFFECT OF VARIOUS SUBSTANCES ON THE ACTIVITY OF ORNITHINE-δ-TRANSAMINASE

Ornithine, α-ketoglutarate, and pyridoxal-5-phosphate were present in all incubations at 35.0, 20.0, and 2.5 μmoles/ml, respectively. The tested substances were present at 25.0 μmoles/ml. The pH was 8.0 in all vessels. Values listed are the means of five samples. Inhibitions listed were significant at $P < 0.05$ in a two tailed t test.

Addition	μmoles PCA produced/mg of protein/hour ± S.E.	% Inhibition
none	22.90 ± 0.728	—
L-hydroxyproline	22.69 ± 0.915	0
L-proline	21.05 ± 0.538	0
L-arginine	24.05 ± 0.919	0
L-isoleucine	20.70 ± 0.994	0
L-leucine	21.72 ± 0.467	0
L-valine	20.64 ± 1.011	0
norvaline	15.78 ± 1.523	31
δ-aminovaleric acid	17.53 ± 1.048	23
α-ketovaleric acid	18.56 ± 1.261	19
hydroxylamine	none detected	100
D-cycloserine	13.35 ± 1.053	42
thiosemicarbazide	none detected	100
INAH (isonicotinamide hydrazide)	9.39 ± 1.188	59

TABLE 5

COMPARISON OF α-KETOGLUTARATE CONCENTRATION IN *FASCIOLA* AND RAT LIVER

Values are in nanomoles of α-ketoglutarate/gram wet weight and each is based on six samples.

Tissue	α-ketoglutarate concentration ± S.E.	Significant difference from *Fasciola*
Fasciola	214 ± 10.0	—
rat liver	54 ± 10.0	$P < 0.001$

We undertook additional studies to test the effectiveness of amino group acceptors other than α-ketoglutarate. In several publications, Kurelec (1974, 1975a, 1975b) has suggested that an ornithine-alanine transaminase is active in the production of proline by *Fasciola*. If so, pyruvate ought to function as an amino group acceptor. Using the purified enzyme and standard enzyme assay conditions (35 mM ornithine, 2.5 mM pyridoxal PO_4, pH 8), we found that neither pyruvate, oxaloacetate, nor glyoxylate (each at 20 mM), is effective in producing measurable PCA. We also examined the possibility that ornithine-alanine transaminase activity might be present in the crude homogenate. Numerous experiments, such as those shown in table 6, indicated that α-ketoglutarate is much more effective than the other amino group acceptors tested and that pyruvate activity can be demonstrated only at concentrations that probably are beyond the physiological range.

DISCUSSION

The pattern of inhibition of *Fasciola* OTA by various structurally related compounds was found to be similar to that reported by Strecker (1965) for rat liver, except that valine, leucine, and isoleucine caused no significant inhibition. Complete inhibition of *Fasciola* OTA was obtained with some inhibitors of pyridoxal-dependent enzymes, indicating that this enzyme, like other transaminases, requires this cofactor. Furthermore, the purification procedure, which undoubtedly resulted in a loss of endogenous pyridoxal, caused a decrease in enzyme activity, which could then be partially restored by the addition of pyridoxal-5-phosphate.

The K_m for ornithine in purified preparations of *Fasciola* OTA is 2.33 mM, a figure similar to that found for rat liver OTA (Strecker, 1965). However, the K_m of α-ketoglutarate for *Fasciola* (2.24 mM) is much higher than that of α-ketoglutarate in rat liver (0.28 mM) (Strecker, 1965). Fluke OTA, there-

TABLE 6

DATA FROM REPRESENTATIVE EXPERIMENTS COMPARING ACTIVITY OF ORNITHINE-δ-TRANSAMINASE IN CRUDE HOMOGENATES OF *FASCIOLA* WITH VARIOUS AMINO GROUP ACCEPTORS

Ornithine and pyridoxal-5-phosphate were present at 35.0 and 2.5 μmoles/ml, respectively. Specific activity values are in μmoles of PCA/mg protein/hour. Each value is the mean of five samples.

Acceptor	Specific activity ± S.E. at various millimolar acceptor conc. 3.75 mM	10.0 mM	75.0 mM
α-ketoglutarate	2.74 ± 0.076	3.45 ± 0.082	not tested
glyoxylate	none detected	0.92 ± 0.043	9.89 ± 0.061
pyruvate	none detected	none detected	0.62 ± 0.070

fore, appears to have a much greater requirement for α-ketoglutarate than either rabbit or rat liver. While mammalian liver OTA may be inhibited at concentrations lower than 10 mM α-ketoglutarate, inhibition of the fluke OTA enzyme was not observed even at 50 mM. It is likely, therefore, that in the fluke the reaction proceeds in the presence of high concentrations of α-ketoglutarate. Measurements made in *Fasciola* support such a conclusion. The source of the α-ketoglutarate may be the highly active glutamic-pyruvic transaminase (GPT) that was demonstrated to occur in *Fasciola* by Connelly and Downey (1968). This enzyme in the worm has eighteen times the activity of its counterpart in liver. The high GPT activity may explain Kurelec's (1974, 1975a) observation that the addition of either arginine or ornithine to *Fasciola* homogenates resulted in increased levels of alanine. Kurelec, however, interpreted his data as indicating that the amino group acceptor for the ornithine transaminase in PCA formation was pyruvate. To support this postulation he presented additional data (Kurelec, 1975a) indicating that the order of formation of compounds involved in proline biosynthesis was proline, alanine, and glutamate. The pattern suggested to Kurelec that the path of the amino group of ornithine is from ornithine to alanine to glutamate. However, in the same paper he reported that α-ketoglutarate was more effective in stimulating proline synthesis than pyruvate when either compound was added with ornithine to *Fasciola* homogenates.

To the present investigators, Kurelec's observations are not inconsistent with α-ketoglutarate as the amino group acceptor in the reaction whereby PCA is produced from ornithine. Furthermore, our data, based on direct measurement of enzyme activity, show that pyruvate is a very poor substrate for PCA formation in *Fasciola*.

Kurelec's observations can be explained by assuming that a highly active GPT, such as that described above, is coupled to the OTA reaction. The GPT would regenerate α-ketoglutarate from the glutamate produced in the OTA reaction. If sufficient ornithine, pyruvate and α-ketoglutarate were available, large amounts of alanine would be produced because of the repeated reconversion of glutamate into α-ketoglutarate in order to meet the high keto acid requirements of the OTA reaction. If this situation occurred in *Fasciola*, the high levels of free alanine found in the worm by Kurelec and Rijavec (1966) would also be explained.

We conclude that *Fasciola* OTA appears to be similar to rat liver OTA in most of the properties studied, except that the fluke enzyme has a greater requirement for α-ketoglutarate. Thus in *Fasciola* it is not only the high specific activity of OTA, but also the high tolerance of this enzyme for α-ketoglutarate, which results in high levels of proline formation.

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